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12 PERSONAL AUTHOR(S)
C.KRISHNAMURTI, C.F.BARR, M ALYCIA HASSET, G.D. YOUNG, AND B.M. ALVING

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Plasminogen Activator Inhibitor: A Regulator of Ancrod-Induced Fibrin Deposition in Rabbits

By Chitra Krishnamurti, Charles F. Barr, M. Alycia Hassett, G. David Young, and Barbara M. Alving

Plasma levels of a fast-acting plasminogen activator inhibitor (PAI), which neutralizes both tissue plasminogen activator (t-PA) and urokinase, are markedly increased in endotoxin-treated rabbits. The ability of this inhibitor to prevent the fibrinolysis that occurs after a thrombogenic stimulus was investigated in a rabbit model. Normal and endotoxin-treated male New Zealand rabbits were infused with ancrod, an enzyme that causes noncrosslinked fibrin formation in vivo. Ancrod stimulated t-PA activity by 90% in normal rabbits and caused hypofibrinogenemia but did not increase PAI levels or induce fibrin deposition in target organs. Rabbits injected with endotoxin (10 µg/kg) showed an increase in PAI from ~1 to 32 U/mL 4 hours later. When ancrod was infused at this time, 90% of the rabbits developed renal fibrin thrombi. Fibrin deposition was recorded in 40% of the rabbits that received a lower dose of endotoxin (1.0 µg/kg) and had a PAI level of 14 U/ml at the time of ancrod infusion 4 hours later. Fibrin deposition did not occur in the endotoxin-treated rabbits that received normal saline. These data suggest that high levels of PAI inhibit fibrinolysis in vivo, thereby promoting fibrin clot deposition following a thrombogenic stimulus.

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TWO CRITICAL FACTORS in the regulation of fibrinolysis in vivo are the functional levels of tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI), both of which are identical to those produced by endothelial cells.[1-3] PAI, a 50,000-mol-wt protein, has an apparent half-life (t½) of 7 minutes when measured in the rabbit circulation[4] and is the major inhibitor of t-PA and urokinase in both humans[5] and rabbits.[4,6] Elevated levels of PAI have been found in plasma from patients who have had venous thromboses,[7,8] gram-negative sepsis,[4,9] and many other conditions predisposing to fibrin clot formation.[10]

Colucci and co-workers[4] demonstrated that infusion of endotoxin into rabbits increases PAI levels in a dose-dependent manner; they postulated that high levels of this inhibitor may have a physiologic role in the coagulopathy associated with endotoxemia. Although other investigators showed that infusion of soluble fibrin into endotoxin-treated rabbits results in renal microclot formation,[11] whether the apparent inhibition of fibrinolysis by endotoxin is mediated through its ability to increase levels of circulating PAI is not known.

The present study used endotoxin-treated rabbits to determine if elevated PAI levels could inhibit the fibrinolysis that occurs after infusion of ancrod, an enzyme that induces non-crosslinked fibrin clots by cleaving fibrinopeptides A, AP, and AY.[12-14] Ancrod causes hypofibrinogenemia and elevated titers of fibrinogen-fibrin degradation products (FDP-fdp) when infused slowly into rabbits and humans.[15,16]

This study demonstrates that ancrod does not cause fibrin deposition in normal rabbits, presumably because of the

fibrinolysis that occurs in response to its initial coagulant action. Ancrod can, however, induce fibrin deposition in animals that have elevated levels of PAI. The data indicate that PAI may promote fibrin formation by preventing the endogenous fibrinolysis that occurs after a thrombogenic stimulus.

MATERIALS AND METHODS

Materials. Purified two-chain human t-PA was purchased from American Diagnostica, Greenwich, CT. The specific activity was 100,000 U/mg, as determined with an international urokinase standard; the data in this article are expressed in terms of this standard. If this preparation of t-PA were calibrated against the International Reference Preparation for t-PA,[17] the specific activity would be ~500,000 IU/mg.[18] Urokinase (specific activity, 100,000 U/mg) was purchased from Abbott Laboratories, North Chicago. Human fibrinogen and Cohn fraction III paste were obtained from Cutter Laboratories, Berkeley, CA. Plasminogen was purified from the paste by affinity chromatography[19] on lysine-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden); the specific activity was 20 CTA U/mg. Human thrombin (H-2) and human plasmin (7.3 U/mg) were supplied by the Office of Biologics, Bethesda, MD. The synthetic substrate D-Val-Leu-Lys-pNA (S-2251) was purchased from Kabi Diagnostica, Stockholm. Ancrod, an enzyme derived from the Malayan pit viper, was the generous gift of Knoll Pharmaceutical, Whippany, NJ, and endotoxin (*Escherichia coli* 0111.B4) was purchased from Difco Laboratories, Detroit.

Animal experiments. Male New Zealand white rabbits weighing between 2.3 and 3.5 kg were received from a single supplier at least 1 week before use and were given standard laboratory food and water ad libitum. Endotoxin was infused into a marginal ear vein during 5 minutes in a total volume of 3 mL of normal saline at a dose of 1.0 or 10.0 µg/kg. Ancrod (2 U/kg) was administered by an infusion pump in a total volume of 20 mL of normal saline into a marginal ear vein during 1 hour. The vasopressin analogue 1-desamino-8-D-arginine vasopressin (DDAVP, Armour Pharmaceutical, Kankakee, IL) was infused into a marginal ear vein during 10 minutes at a dose of 0.4 µg/kg in 10 mL of normal saline. Blood samples (0.9 mL) were collected from the uninfused marginal ear vein into polypropylene tubes that contained 0.1 mL of 2% EDTA and immediately centrifuged for 1 minute at 22 °C in an Eppendorf centrifuge 3200 (Brinkman Instruments, Westbury, NY). The plasma was immediately separated from the RBCs, and aliquots were stored at −70 °C or acidified in preparation for measurement of plasminogen activator.

Assay of plasminogen activator activity. Assay for plasminogen activator activity was performed according to the method of Wiman

and colleagues.[20] Plasma was acidified with an equal volume of 1.0 mol/L of sodium acetate pH 3.9 and incubated at 22 °C for 15 minutes. An aliquot of this mixture was diluted to a final plasma concentration of 0.5% in 0.05 mol/L of Tris, 0.1 mol/L NaCl, and 0.1% Triton X-100 pH 8.8 (NEN, Boston), and incubated with 1 mmol/L of S-2251 and 0.3 mg/mL of plasminogen in the presence of fibrin (70 μg/mL) for 6 hours at 37 °C in a microtiter plate. The change in absorbance was measured at 405 nm with a Titertek Multiscan Spectrophotometer (Flow Lab, McLean, VA).

Assay of PAI activity. The PAI assay was performed as described by Chmielewska and colleagues.[21] Human t-PA (10 μL, 2U/mL final) was incubated for 10 minutes at 22 °C with 40 μL of plasma that was undiluted or diluted in 0.02 mol/L of sodium phosphate, 0.1 mol/L of NaCl pH 7.3. Residual t-PA activity was measured after acidification and dilution of the sample as described previously. One unit of inhibitor is defined as the amount that inactivates 1 U of t-PA during the 10-minute incubation period.

Assay of plasmin-inhibitor activity. Plasmin (final concentration 0.6 CTA U/mL) was incubated for 20 seconds at 30 °C with rabbit plasmas diluted 1/20 and 3/40 in 0.05 mol/L of Tris and 0.15 mol/L of NaCl pH 7.4. After 20 seconds, a 30-μL sample was added to 1 mL of 0.1 mmol/L of S-2251 in 0.05 mol/L of Tris pH 7.4, and the change in absorbance at 405 nm was monitored with a Cary recording spectrophotometer (Varian Associates, Instrument Division, Palo Alto, CA). The results were expressed as the percentage of activity of a standard of pooled human plasma.

Plasma plasminogen assay. Plasma was mixed with an equal volume of 1.0 mol/L of sodium acetate pH 3.9 and diluted to final concentrations of 2% and 1% with 0.05 mol/L of Tris, 0.1 mol/L of lysine, and 0.1% human albumin, pH 7.4. A 200-μL mixture containing 0.05% to 2.5% plasma, urokinase (2,500 U), S-2251 (0.2 mmol/L) in Tris-lysine buffer was then incubated in a microtiter plate for 30 minutes at 22 °C, and the change in absorbance was compared with that obtained with a standard curve prepared from human plasmin of known activity.

Fibrin plate zymography. Plasma samples were electrophoresed in sodium dodecyl sulfate (SDS) on an 8% polyacrylamide gel that was then washed for 30 minutes in 0.1 mol/L of Tris pH 8 containing 0.1% Triton X-100.[22] The gel was overlaid with a fibrin monolayer containing plasminogen, incubated at 37 °C for 16 hours, and photographed.[22]

Fibrinogen and FDP-fdp. The fibrinogen concentration was determined spectrophotometrically after plasma was clotted with a calcium-thrombin solution for 1 hour, and the clot was washed with normal saline followed by solubilization in alkaline urea.[15] The FDP-fdp were measured in a tanned RBC hemagglutination immunoassay that was modified for use in rabbits.[15]

Data analysis. The results of experiments were expressed as the mean + SEM. Statistical analysis was performed with a Student's *t* test on paired samples.

Table 1. Measurement of PA Activity in Rabbit Plasma

Rabbit No.	PA (U/mL)		
	A	B	C
I	0.41	0.42	0.09
II	0.66	0.66	0.42
III	0.55	0.51	0.23
IV	0.74	0.60	0.34
Mean · SEM	0.59 ± 0.07	0.55 ± 0.05	0.27 ± 0.07

Plasma was acidified, diluted to 0.5%, and assayed immediately (A); acidified, diluted, frozen at 70 °C for 24 hours and then thawed and assayed (B); acidified, frozen at 70 °C for 24 hours and then diluted and assayed (C).

Histopathology. Necropsies were performed on animals that died during ancrod infusion or were killed by a 5-mL intravenous (IV) injection of sodium pentobarbital (V-Pento C, 65 mg/mL) 1 hour after completion of the infusion. Within 20 minutes of death, tissues from all animals were immersion-fixed in neutral buffered 10% formalin, processed, embedded in paraffin, and stained with hematoxylin and eosin. Sections of lung, liver, kidney, and spleen were also stained with phosphotungstic acid hematoxylin (PTAH)[23] and examined for the presence of fibrin microthrombi by a pathologist who was unaware of the experimental protocol for the individual animals.

RESULTS

Measurement and characterization of PA in rabbit plasma. Although freezing acidified human plasma has been reported not to affect the PA activity,[20] freezing and storage of rabbit plasma at -70 °C caused a significant decrease in PA activity as compared with that of unfrozen samples (Table 1). When acidified rabbit plasma was diluted to a final plasma concentration of 0.5% in Triton X-100 buffer and then stored at -70 °C, the PA remained unchanged from that measured in fresh plasma. Therefore, all plasmas were stored at -70 °C after acidification and dilution in Triton X-100 buffer. The apparent lability of rabbit PA could not be explained on the basis of qualitative or quantitative alterations in activators or inhibitors. Both species have similar levels of PAI and PA[6]; by fibrin zymography, the mol wt of PA in rabbit plasma is 68,000, similar to that of human t-PA.[6] The PAI in rabbit plasma has the same mol wt as the PAI that has been characterized in human plasma.[4]

Effects of ancrod infusion in normal rabbits. When ancrod was infused into normal rabbits, fibrinogen levels decreased from 2.2 ± 0.1 to 0.3 ± 0.1 g/L and FDP-fdp increased from a baseline titer of 1/32 to 1/4098 at 1.5 hours after initiation of the infusion. These alterations were associated with a significant increase in PA activity of 91.3% ± 16.1% (*P* < .01, Fig 1). Six hours later, the PA levels had returned to baseline values, and the fibrinogen levels were still depressed (0.4 g/L). When ancrod was infused at this time into these hypofibrinogenemic rabbits, PA activity was again increased by 40.7% ± 14.6% above the 6-hour value
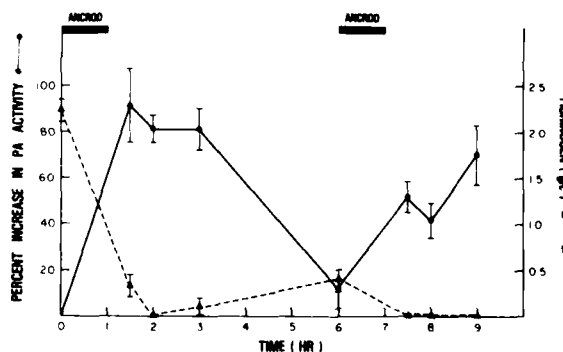

Fig 1. Effect of ancrod on plasminogen activator (PA) activity in hypofibrinogenemic rabbits. Ancrod (2 U/kg) was infused at 0 and 6 hours. PA activity (———) and fibrinogen levels (— — —) were determined at the indicated time points. Data are the mean + SEM of four rabbits.
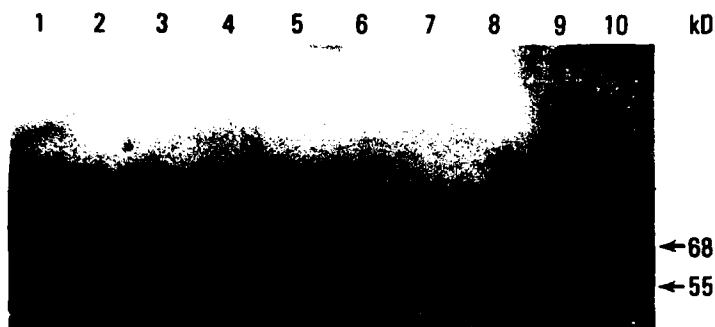
Fig 2. Fibrin zymogram of paired rabbit plasmas obtained before and 1.5 hours after infusion of ancrod or saline. Samples were electrophoresed in sodium dodecyl sulfate (SDS) on an 8% polyacrylamide gel. Lanes 1,3, pre-ancrod samples, lanes 2,4, post-ancrod; lanes 5,7, pre-saline; lanes 6,8, post-saline; lane 9, human-melanoma tissue plasminogen activator (t-PA); lane 10, human urokinase.

($P < .05$) and fibrinogen levels became undetectable. The PA values remained elevated for another 2 hours after the second ancrod infusion. By fibrin plate zymography, the PA before and after ancrod was 68,000 mol wt (Fig 2), suggesting that the increase in PA after ancrod was t-PA. Ancrod had no effect on PAI levels, which were <1 U/mL when measured before and at 1.5 hours after infusion. Infusions of saline into six control rabbits caused no increase in PA, PAI, or change in fibrinogen levels (data not shown).

To determine if ancrod could stimulate release of PA in the absence of any detectable fibrinogen, a group of animals was rendered afibrinogenemic by infusing ancrod at 0 to 1 hour and again at 2 to 3 hours (Fig 3). At 6 hours, these afibrinogenemic rabbits received either a third infusion of ancrod or DDAVP, which is known to stimulate PA in normal rabbits by 40%.[6] Ancrod caused no increase in PA when infused at this time; however, PA was significantly increased by 26% ($P < .05$) in the five rabbits that had received DDAVP. These data indicate that ancrod stimulates PA release only when infused into rabbits that have circulating fibrinogen, suggesting that clot formation induced by ancrod is the stimulus for PA release. The ability of the afibrinogenemic rabbits to release PA when infused with DDAVP indicates that the PA stores had not been completely depleted by two previous infusions of ancrod.

Ancrod-induced fibrin deposition in endotoxin-treated rabbits. Infusion of endotoxin into rabbits caused a dose-dependent increase in both PA and PAI (Fig 4A and B). One group of rabbits received endotoxin at a dose of 1 μg/kg, followed by an infusion of normal saline or ancrod 4 hours later (Fig 4A). Approximately 90 minutes after endotoxin infusion, PA was maximally increased by 41.2% $\pm$ 10.5% above baseline values ($P < .01$), returning to normal values by 4 hours. PAI levels increased from <1 U/mL to maximum values of 14.3 $\pm$ 2.3 U/mL at 4 hours. Infusion of ancrod at this time did not cause an increase in PA, although fibrinogen levels decreased from 1.7 $\pm$ 0.3 to 0.1 $\pm$ 0.1 g/L. At necropsy, 40% of the rabbits had evidence of renal thrombi (Table 2). One of these animals also had pulmonary thrombi. None of the endotoxin-treated animals that received saline instead of ancrod showed any evidence of fibrin deposition.

A second group of rabbits was infused with endotoxin at a dose of 10 μg/kg, followed by ancrod or normal saline 4 hours later. After infusion of endotoxin, PA was maximal at

30 minutes and declined at 90 minutes as PAI levels rapidly increased (Fig 4B). By 4 hours, PAI had increased from <1 to 32 U/mL; plasminogen levels had decreased from 5.9 to 4.2 U/mL, and α_2-antiplasmin was decreased by 36%. Thus the most striking change in components of the fibrinolytic system 4 hours after this dose of endotoxin was the marked increase in PAI. Infusion of ancrod at this time caused glomerular fibrin deposition in 90% of the animals (Table 2). One of these animals also had pulmonary thrombi, and one demonstrated pulmonary and splenic thrombi in addition to the fibrin deposition in the glomeruli (Fig 5). The endotoxin-treated animals that received saline had no demonstrable fibrin deposition.

These data suggested that endotoxin, by increasing PAI levels, promoted fibrin deposition in the ancrod-treated rabbits. The following experiment was done to determine if ancrod induced clot deposition when infused into endotoxin-treated rabbits that had PAI levels <1 U/mL at the time of initiation of infusion. Ancrod was infused into rabbits 30 minutes after they had received endotoxin (10 μg/kg). PAI levels increased from <1 to ~16 U/mL during the ancrod infusion and were 24 U/mL 1 hour later, at the time of necropsy. Fibrin deposition was detected in 50% of these rabbits (Table 2).

All rabbits infused with ancrod alone or with endotoxin at a dose of 1.0 μg/kg followed by ancrod survived the infusion.
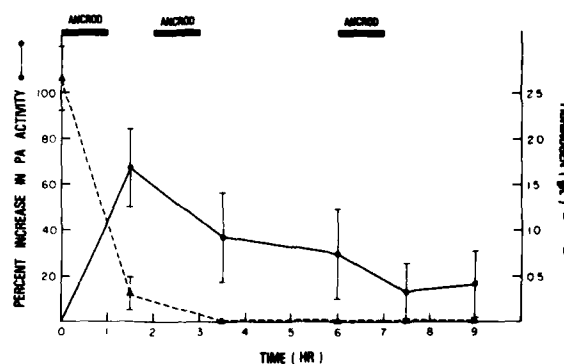

Fig 3. Fibrinolytic response in rabbits after multiple infusions of ancrod. Plasminogen activator (PA) activity (———) and fibrinogen levels (— — —) were measured at the indicated time intervals after ancrod infusions at 0, 2 and 6 hours. Results are the mean $\pm$ SEM of five rabbits.

Fig 4. Effect of ancrod on endotoxin-treated rabbits. Endotoxin was administered at doses of 1 μg/kg (A, 12 rabbits) or 10 μg/kg (B, 13 rabbits), and ancrod was infused 4 hours later. The plasminogen activator (PA) (———) and PA inhibitor (PAI) (— — —) activities (mean ± SEM) are shown.
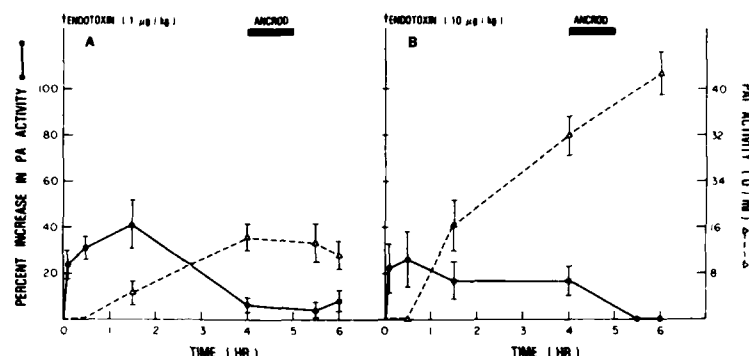
Animals that received endotoxin at the higher dose of 10 μg/kg followed by ancrod at 30 minutes or 4 hours had mortalities of 50% and 40%, respectively. The mortality was 14% for those animals that received only saline 4 hours after this dose of endotoxin. Mortality could not be correlated with the presence of fibrin deposition.

DISCUSSION

The present study demonstrates that when a thrombogenic stimulus such as ancrod is infused slowly into normal rabbits, PA levels increase as a result of the coagulant action of ancrod on fibrinogen. PAI levels are not increased and fibrin deposition does not occur after ancrod infusion alone. However, rabbits that have a high level of PAI at the initiation of ancrod administration or rabbits that are undergoing rapid increases in PAI during the infusion may develop fibrin

Table 2. Summary of Histologic Studies

	Fibrin Thrombi		
Study/Treatment	(%)	(No. of Animals)	PAI Levels (U/mL)* (Mean + SEM)
Experimental			
Endotoxin (1 μg/kg), ancrod (4 h)†	40%	(2/5)†	11.7 · 3.1
Endotoxin (10 μg/kg), ancrod (4 h)	90%	(9/10)	33.3 · 3.4
Endotoxin (10 μg/kg), ancrod (30 min)	50%	(3/6)	0.2 · 0.1
			24.7 · 3.4
Controls			
Endotoxin (1 μg/kg), saline (4 h)	0%	(0/4)	20.4 · 6.0
Endotoxin (10 μg/kg), saline (4 h)	0%	(0/7)	33.3 · 2.9
Saline, ancrod (4 h)	0%	(0/4)	2.0 · 1.6

*Plasminogen activator inhibitor (PAI) levels at time of second infusion (ancrod or saline). For rabbits that received ancrod 30 minutes after endotoxin, PAI levels had not yet reached a plateau and increased from 0.2 to 24.7 U/mL at time of necropsy.

†Time of second infusion (ancrod or saline). After the administration of endotoxin, ancrod or saline was infused into the animals. Survivors were killed 1 hour postinfusion. Lung, liver, spleen, and kidneys were fixed, stained with phosphotungstic acid hematoxylin (PTAH), and examined for the presence of fibrin microthrombi.
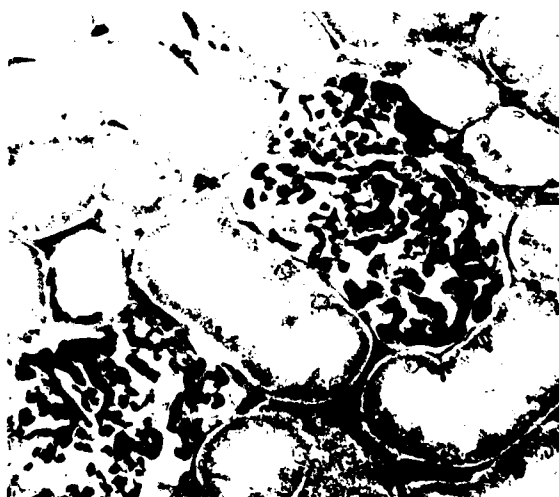
deposition, predominantly in the glomeruli. It is unlikely that alterations in other components of the fibrinolytic system are sufficient to be implicated in the thrombus formation that occurred in endotoxin-treated rabbits that received ancrod. Although plasminogen levels decreased after endotoxin infusion, α_2-antiplasmin levels were lower as well.

The infusion of endotoxin also causes leukopenia, generation of cellular and humoral procoagulant activity, and activation of the complement system.[24] These alterations, however, do not appear to be significant factors in the induction of fibrin formation described in the present study. In their work with rabbits deficient in the sixth component of complement, Muller-Berghaus and Lohmann demonstrated that the complement system does not have a role in the induction of renal microclot formation in animals that receive a continuous infusion of endotoxin.[25] Muller-Berghaus and coworkers also reported that rabbits injected simultaneously with ancrod and endotoxin developed glomerular microclots that could not be prevented by heparin[26] or by first rendering the animals neutropenic with nitrogen mustard.[27] They concluded that the endotoxin-mediated generation of

Fig 5. Histopathologic evidence of fibrin microthrombi (dark, fibrillar areas) in the glomeruli of a rabbit infused with endotoxin (10 μg/kg) and then ancrod 4 hours later. The animal was killed 1 hour after completion of ancrod infusion.

microclots in ancrod-infused rabbit did not involve neutrophils or the generation of procoagulant activity by endotoxin.

Additional indirect evidence for the role of PAI in inhibiting fibrinolysis is suggested by the studies of Szczepanski and Lucer,[28] who reported that the infusion of a single dose of endotoxin into rabbits caused an increase in the fibrinolytic activity of the plasma euglobulin fraction within the first hour, followed by a decrease in the plasminogen activator activity of kidney tissue at ~6 hours. Bergstein and Michael[29] found that fibrinolytic activity was present in the renal cortices of rabbits killed 30 minutes after a single dose of endotoxin but was undetectable in animals killed from 1 .. hours later. The activity reappeared at 24 hours, thus following a time course consistent with induction of PAI. The loss of fibrinolytic activity after infusion of endotoxin alone was not associated with fibrin deposition.

The vulnerability of the kidneys of the endotoxin-treated rabbits to fibrin deposition after ancrod may reflect the low level of PA activity relative to the levels of PAI within this particular organ. In the present study, only 3 of 14 rabbits that developed renal fibrin deposition had pulmonary thrombi as well. One explanation for these findings is that

pulmonary tissue can have an inhibitory effect on the function of PAI, as was recently demonstrated by Colucci and co-workers.[18] These investigators showed that addition of rabbit lung slices to a whole blood clot markedly enhanced its lysis when it was incubated in the presence of PAI inhibitor-rich plasma in vitro.

The highest levels of PAI appear to be induced by endotoxin in humans[4,9] as well as in rabbits. However, the levels of PAI required to prevent fibrinolysis are not known for specific clinical settings. The levels may depend on the type of thrombogenic stimulus that is acting in a given situation. For example, ancrod induces non-crosslinked fibrin formation which undergoes lysis relatively easily. The generation of small amounts of thrombin which causes activation of coagulation factor XIII with crosslinking of α_2-antiplasmin to the clot[30] may be much more thrombogenic and require larger concentrations of activator for lysis.

Elevated PAI levels appear to be a significant factor for clot formation when a thrombogenic stimulus such as ancrod is administered. Monitoring levels of this inhibitor in patients, especially those in sepsis, may allow prevention of renal fibrin deposition, which is not uncommon in this clinical condition.[31]

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